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In the course of our studies on the immunotherapy of gram-negative bacterial sepsis, we devised an animal model of sepsis which closely mimicked the clinical presentation of sepsis in humans in both the kinetics and level of bacteremia observed as well as in the cytokine profile generated. Additionally, we observed in preliminary experiments that a J5 antiserum prepared from a whole cell vaccine administered to rabbits was protective in this neutropenic rat model of sepsis. Using this model, we have assessed the ability of antibody fractions directed against the J5 E. coli to protect against lethal Pseudomonas aeruginosa sepsis and examined in greater detail the preparation of J5 vaccines.

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Novel Therapeutic Strategies in the Treatment of Sepsis

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New Trends in *Escherichia coli* O111:B4 J5 Mutant (R_c Chemotype) Vaccine Development for Use in Gram-Negative Bacillary Sepsis

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I. INTRODUCTION

With the recognition that there was still an unacceptably high mortality rate from bacterial sepsis despite the introduction of new, potent antimicrobial agents, many investigators examined the potential of immunotherapeutic strategies to supplement the conventional treatment of this condition. This effort was facilitated by the elucidation of the structure of lipopolysaccharide (LPS) by both biochemists and bacterial geneticists. Lipid A and inner core sugar regions of the molecule were found to be widely shared by a diverse array of gram-negative bacilli, and that an outer polysaccharide region of the molecule, the 0 repeat region, provided serological specificity to the individual strains of bacteria.

Chedid et al. (1–3) and colleagues provided important data in animal models which suggested that immunization with vaccines in which the core LPS regions (i.e., "rough" LPS) were exposed could provide protection against challenge with heterologous organisms.

Further work with one of these rough LPS vaccines, the *Escherichia coli* O111:B4, J5 (R_c chemotype) mutant, led Ziegler, McCutchan, and colleagues to test the efficacy of anti-J5 antisera in patients suspected of having gram-negative bacterial sepsis (4). In this study, administration of J5 antisera led to a significant decrease in mortality, particularly in the subgroup of patients who required pressor agents for at least 6 h; however, protection did not correlate with hemagglutinating antibody titers. Moreover, since J5 antisera was prepared in normal volunteers immunized with a boiled whole-cell vaccine, the nature of the protective antigen was not clear.

The inability to determine either the protective antigen or the role of antibody in the protection observed may have contributed to the failure of investigators to confirm these results in subsequent clinical studies (5,6). Consequently, the validity of the hypothesis that antibody to the inner core region of LPS might protect against a wide array of gram-negative bacterial pathogens has been questioned (7,8). In addition, the lack of an adequate animal model made it difficult to evaluate potential therapeutic candidates before undertaking clinical trials.

In the course of our studies on the immunotherapy of gram-negative bacterial sepsis, we devised an animal model of sepsis which closely mimicked the clinical presentation of sepsis in humans both in the kinetics and level of bacteremia observed as well as in the cytokine profile generated (9). Additionally, we observed in preliminary experiments that a J5 antisera prepared from a whole-cell vaccine administered to rabbits was protective in this neutropenic rat model of sepsis (10). Using this animal model of sepsis, we assessed the ability of antibody fractions directed against the J5 *E. coli* to protect against lethal *Pseudomonas aeruginosa* sepsis (11), and examined in greater detail the preparation of J5 vaccines.

II.   NEUTROPENIC RAT MODEL OF SEPSIS

Although Ziegler and Douglas developed a neutropenic rabbit model of sepsis that mimicked the pathogenesis of sepsis in humans in both the route of infection and inoculum size, the size and cost of rabbits made it difficult to perform experiments with sufficient numbers of animals for statistical validity (12). We previously reported the development of a neutropenic rat model of infection that also employed a modest inoculum typical of human exposure to infection (9). Briefly, Sprague-Dawley rats weighing 200–300 g were pretreated with cefamandole intramuscularly to alter the normal rat gut flora. The rats were given cyclophosphamide at time 0 (150 mg/kg) and at 7 hr (75 mg/kg). The neutropenia

thus engendered reached a nadir at day 5 and remained low until day 9, after which the white blood cell counts recovered. Bacteria were fed orally to the rats at time 0 and at 48 and 96 h. The animals were followed for the development of fever with an infrared temperature probe. Treatment was administered intravenously to animals whose temperature was $> 38°C$ (usually day 5). These animals typically had bacteremia with the challenge organism. Animals were followed for 12 days, after which time the white blood cell counts returned to normal and deaths no longer occurred.

III. *ESCHERICHIA COLI* J5 KILLED WHOLE-CELL VACCINE

A. Method of Preparation

While investigators have focused on the source of the J5 isolate used for the preparation of a vaccine, relatively few have carefully examined the manner in which the vaccine is prepared. We originally obtained a culture of *E. coli* O111:B4, J5 mutant from Dr. Elizabeth Ziegler, and kept it frozen at $-70°C$. Since the J5 organism has been described as a ''leaky'' mutant (13), we streaked the J5 culture for single-colony isolation on Trypticase soy agar. We then selected 5–7 colonies, which were then individually subcultured without shaking on Trypticase soy broth (TSB) overnight at 37°C. On the following day we chose that subculture that had the clearest supernatant, indicative of the isolate with the ''roughest'' LPS. This subculture was then used to inoculate a 2-L flask containing 1 L of TSB for overnight stationary culture. The following day the bacteria were harvested, washed, and resuspended in saline to a stock optical density (OD) of 2.0, which usually corresponded to a colony count of 1×10^{10} CFU/mL. After samples were obtained to confirm the colony counts, the stock culture was boiled for 1 h, which resulted in complete loss of viability of the culture.

For immunization studies, dilutions of the stock culture were made in saline to achieve a 70% light transmission at 610 nM as was done earlier by Ziegler et al. (12). Alternatively, the boiled vaccine was lyophilized and later administered on a weight basis.

B. Immunization of Rabbits

A suspension of killed *E. coli* J5 whole-cell vaccine at a concentration of 10^{10} CFU/mL was used for immunization of rabbits. Rabbits received a series of primary intravenous immunizations on Mondays, Wednesdays and Fridays for 3 weeks. Doses on the respective days were 0.1, 0.1, and 0.2 mL during the first week; 0.2, 0.2, and 0.4 mL during the second week; and 0.8, 0.8, and 1.6 mL during the third week. Blood samples were collected 10 and 47 days after the primary series of immunizations. Booster injections with 1.0 mL of vaccine

were given at approximately 6-week intervals, and about 30 mL of blood was collected 10–14 days after each booster injection. Serum samples were analyzed by enzyme linked immunosorbent assay (ELISA) (14).

ELISA titer of postbleed rabbit serum against J5 LPS 10 days after the primary series of immunizations was 102-fold higher than the prebleed serum (Table 1). The titer of postbleed serum against *E. coli* lipid A was 25-fold higher than the prebleed serum. The ELISA titers dropped to about one-third that level at 47 days post the primary series of immunizations. Booster injection brought the anti-J5 LPS titer close to the 10-day level, but the anti-lipid A titer was about twofold higher than before. Subsequent booster immunizations maintained the high titers against both J5 LPS and lipid A (Table 1).

IV. PURIFIED IMMUNOGLOBULINS AS PROTECTIVE ANTIBODIES

A. Fractionation of Rabbit Sera

The immune rabbit serum was fractionated into purified IgG, IgM, J5 LPS-specific IgG, and non-J5 LPS-specific IgG by affinity chromatography (Fig. 1). In this procedure the whole serum was cycled through protein G-Sepharose immunoadsorbent, which retained most of the IgG present in the serum (Fig. 1, step 1). A small amount of IgG not retained by this column was separated by chromatography on anti-rabbit IgG-Sepharose 4B immunoadsorbent (Fig. 1, step

Table 1 ELISA Titers of Sera from Rabbit #9 (R#9) Immunized with Heat-Killed *E. coli* J5 Whole-Cell Vaccine

Serum description	Days after last		Titers in OD units	
	Primary injection	Booster injection	J5 LPS	Lipid A
Prebleed serum pool	—	—	190	388
Postimmune bleed 1	10	—	19,430	5,900
2	47	—	5,158	2,140
3	68	21	15,628	9,164
4	94	47	10,368	5,491
5	138	14	14,451	15,616
6	151	27	9,676	12,518
7	184	18	14,592	19,040
8	208	10	9,292	11,852

Note: Booster injections were given between bleeds 2 and 3, 4 and 5, 6 and 7, and 7 and 8.
OD = optical density.
Data from Ref. 11.

2). The nonadsorbed serum from step 2 contained IgM and other serum proteins but was free of IgG. Purified IgG was eluted from the protein G-Sepharose column with 0.15 M glycine:HCl buffer pH 2.50 (Fig. 1, step 3). The purified IgG was further fractionated into J5 LPS-specific IgG by chromatography on J5 LPS-EAH-Sepharose 4B immunoadsorbent (Fig. 1, step 4). The IgG not retained by this column is the non-J5 LPS-specific IgG. An alternative scheme for the preparation of purified IgM free of IgG and other serum proteins was successful using size-exclusion chromatography of rabbit serum on a TSK 3000-SW preparative high-performance liquid chromatography (HPLC) column, whereby the IgM elutes at the void volume as the first peak (Fig. 2). A small amount of IgG aggregates co-elutes with IgM and can be removed by affinity chromatography on anti-rabbit IgG-Sepharose 4B as shown in Fig. 1, step 2. Purified IgG was also prepared from the preimmune rabbit serum by affinity chromatography as shown in Fig. 1.

B. Protection of Neutropenic Rats

Protection experiments in neutropenic rats against lethal challenge with a virulent strain of *Pseudomonas aeruginosa* #12:4:4 (Fisher Devlin immunotype 6)
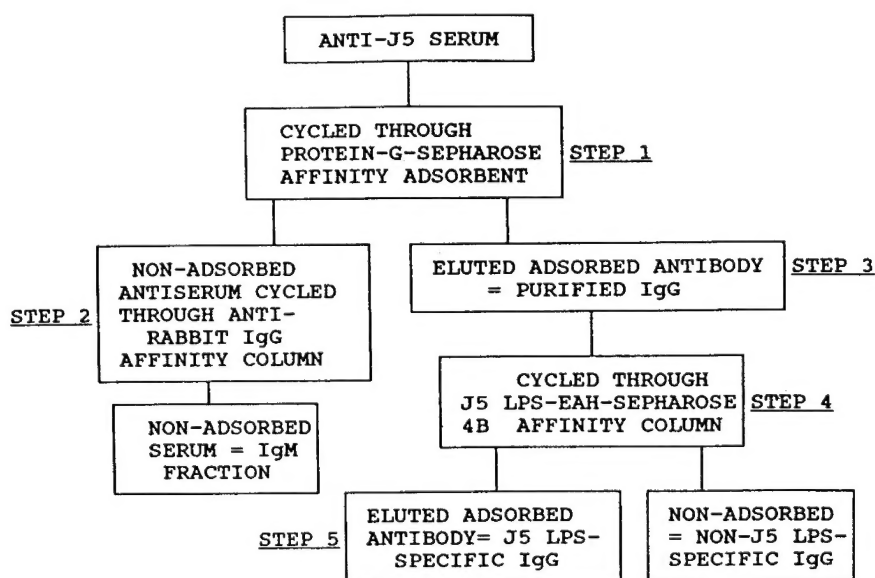

Figure 1 Scheme for the preparation of purified IgG, IgM, and J5 LPS-specific IgG from anti-J5 rabbit serum. The IgM fraction obtained after step 2 was free of IgG but contained other serum proteins. (From Ref. 11.)
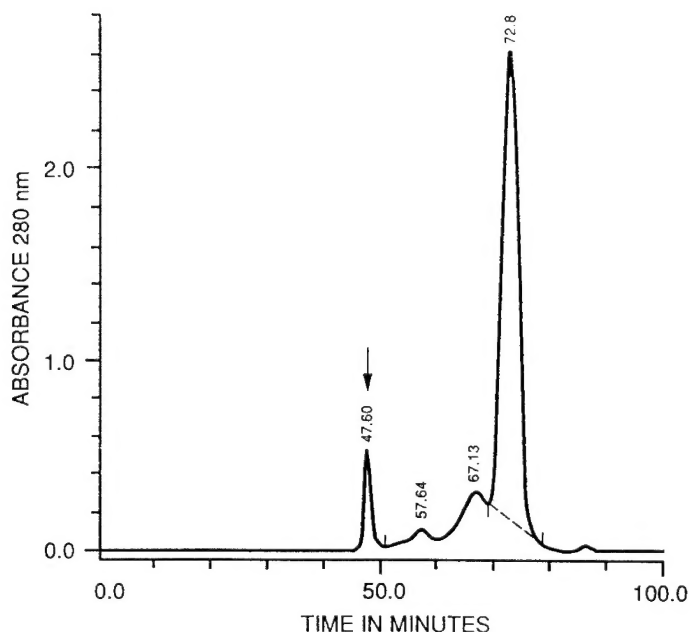
Figure 2 Fractionation of rabbit serum by preparative HPLC on TSK-3000-SW column (22.5 × 600 mm). Elution was performed with 0.01 M Na-phosphate, 0.14 M NaCl, pH 7.4 (PBS) at a flow rate of 2.0 mL/min. The arrow indicates the position of the void volume of this column.

showed that J5 LPS-specific IgG at a dose of 130 µg per rat protected 6 of 8 rats (75%) compared to none of 25 rats treated with IgG (1500 µg per rat) prepared from the preimmune rabbit serum ($p < 0.001$). The unfractionated postimmune IgG at a dose of 2100 µg per rat protected 13 of 20 rats (65%), and the non-J5 LPS-specific IgG at a dose of 2830 µg per rat protected 4 of 13 rats (30%, Fig. 3). These results showed that the J5 LPS-specific IgG was highly protective in this neutropenic rat model of sepsis. In a separate experiment, 9 of 16 rats (56%) were protected when treated with the IgM fraction. This IgM fraction was free of IgG but contained other serum proteins.

V. SUBUNIT VACCINE FORMULATIONS USING PURIFIED *ESCHERICHIA COLI* J5 LIPOPOLYSACCHARIDE

A. Covalent Conjugate with Diphtheria Toxoid

Escherichia coli J5 LPS used alone as vaccine showed only a two- to fourfold rise in anti-J5 LPS antibodies. Therefore, J5 LPS was covalently linked to
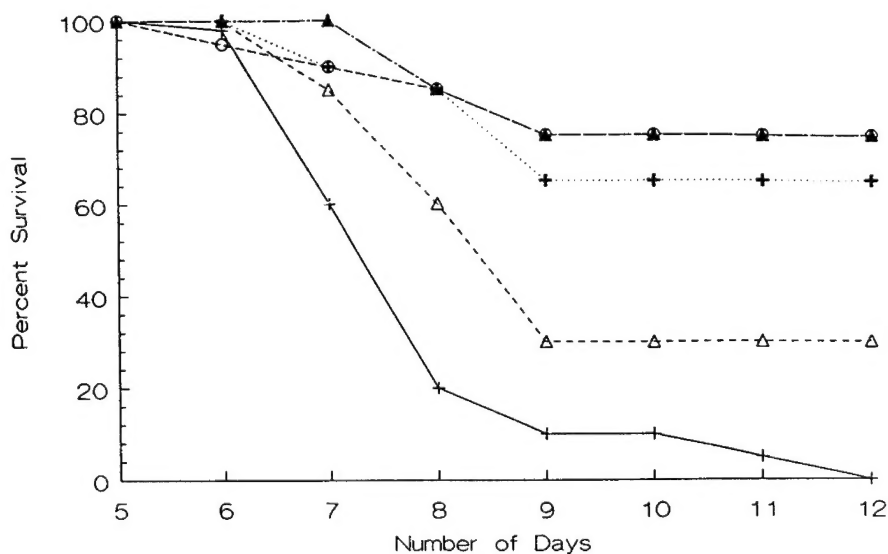
Figure 3 Survival of neutropenic rats against challenge with *Pseudomonas aeruginosa* strain 12:4:4. The antibodies were administered at 9.0 mL/kg. +, IgG from preimmune rabbit serum (1mg/mL); △, non-J5 LPS-specific IgG (1.4 mg/mL); ○, anti-J5 sera; +, purified IgG (1.4 mg/mL); ▲, J5 LPS-specific IgG (96 µg/mL) (From Ref. 11.)

diphtheria toxoid (Dt) by carbodiimide-mediated coupling reaction (15) to give the J5 LPS-Dt covalent conjugate. The molar ratio of J5 LPS:Dt was 5:1. Rabbits were immunized intramuscularly with this vaccine, with and without alum as adjuvant. The conjugate with alum gave only a three- to ninefold rise in titer of anti-J5 LPS antibodies as determined by ELISA. The vaccine given alone in sterile 0.9% NaCl gave a seven- to 16-fold rise in titer against J5 LPS after two doses of vaccine given 4 weeks apart (Table 2). There was only a two- to fourfold rise in titer against *E. coli* lipid A (data not shown). The reason for the vaccine with alum showing weaker anti-J5 antibody response than the J5 LPS-Dt conjugate alone is not clear. In a separate study we found that *Brucella* LPS with alum gave significantly higher antibody response in mice when given subcutaneously than when given intraperitoneally (data not shown).

B. Noncovalent Complex with *Neisseria meningitidis* group B Outer Membrane Protein

A noncovalent complex vaccine formulation was prepared with purified J5 LPS and *Neisseria meningitidis* group B outer membrane protein (GBOMP) (16).

Table 2   ELISA IgG Titers Versus J5 LPS of Sera from Rabbits Immunized with J5 LPS-Dt Covalent Conjugate

Rabbit	ELISA titers in OD units			
	Prebleed	Post-1	Post-2	Fold rise
56	60	130	172	2.8
57	35	128	324	9.2
58	75	397	527	7.0
59	43	832	697	16.2

Rabbits 56 and 57 were immunized with the vaccine containing alum as adjuvant. Rabbits 58 and 59 were immunized with the vaccine without alum. Post-1 = 4 weeks post primary immunization. Post-2 = 1 week post secondary immunization.

Rabbits immunized with the J5 LPS-GBOMP vaccine in sterile 0.9% NaCl elicited high-titer anti-J5 LPS antibodies (Table 3). There was a 37- to 142-fold rise in titer 4 weeks after the first injection, and a further three- to sixfold rise in anti-J5 LPS antibody titer 7 days after the second injection. These rabbits did not show any significant rise in anti-lipid A antibodies. The immune rabbit serum protected neutropenic rats against lethal challenge with *Pseudomonas aeruginosa* strain 12:4:4. Six of 10 rats treated with the postimmune whole serum survived, compared to none of 10 rats treated with the preimmune serum from the same rabbit ($p < 0.01$, Fig. 4). *Neisseria meningitidis* GBOMP is a highly hydrophobic protein that is insoluble in aqueous solvents without added detergent (17). In the noncovalent complex formulation with LPS, the detergent is slowly replaced such that the LPS can bind to the protein by hydrophobic interaction. In successful complex formation the hydrophilic parts are exposed, making the complex soluble in aqueous solvents. This process also seem to result in optimal presentation of the protective J5 LPS epitope.

Table 3   ELISA IgG Titers Versus J5 LPS of Sera from Rabbits Immunized with J5 LPS-GBOMP Noncovalent Complex Vaccine

Rabbit	ELISA titers in OD units			
	Prebleed	Post-1	Post-2	Fold rise
62	106	3,995	25,804	240
63	99	4,115	14,873	147
64	32	3,558	N.D.	—
65	32	4,550	16,384	511

Post-1 = 4 weeks post primary immunization. Post-2 = 1 week post secondary immunization. N.D. = not done.
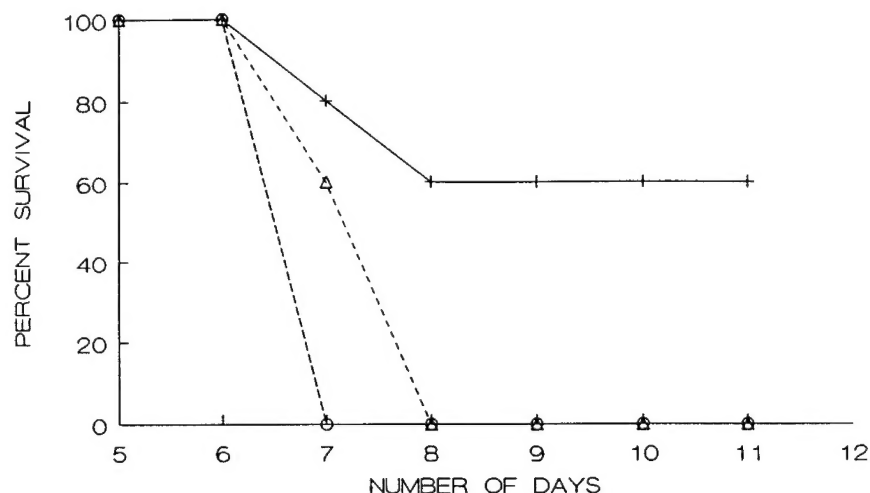
Figure 4 Protection of neutropenic rats against lethal challenge with *Pseudomonas aeruginosa* strain 12:4:4. The antibodies were administered at 9.0 mL/kg. +, postimmune serum of rabbit immunized with J5 LPS-GBOMP vaccine; △, preimmune rabbit serum; ○, PBS control.

VI. CLINICAL SIGNIFICANCE AND FUTURE DIRECTIONS

These data demonstrate that IgG antibody directed toward a J5 epitope, when administered at the onset of fever, is able to protect neutropenic rats from lethal experimental *Pseudomonas* sepsis. The mechanism of this protection is not clear; however, this antibody does not appear to neutralize the biologic activity of endotoxin directly. Preliminary data suggest that it may facilitate the clearance of endotoxin, but this remains to be tested more directly.

It is also necessary to determine the extent to which these anti-J5 antibodies bind to other gram-negative bacteria. When analyzed by flow cytometry, affinity-purified anti-J5 IgG binds to clinical isolates of *E. coli*, *Klebsiella*, and *Enterobacter*, but not to gram-positive cocci (data not shown). IgG prepared from pre-J5 immunization sera did not bind to the gram-negative bacteria. It is necessary to test the protective efficacy of anti-J5 IgG in neutropenic rats that are challenged with gram-negative bacterial pathogens other than *Pseudomonas*.

While our studies in experimental animal models of infection demonstrated the protective efficacy of anti-J5 antibodies, recent clinical studies with J5 antisera and with a J5 hyperimmune IVIG failed to show any benefit (5,6). Before further development of a J5 vaccine, it will be important to resolve the reasons for the failure of anti-J5 therapy in these studies. Our initial studies with the boiled, whole-cell vaccine used sera harvested after booster immunizations for antibody preparation. While the affinity of anti-J5 antibody was not measured, it may be

that antibody elicited following booster doses of a J5 vaccine might have a higher affinity than antibody harvested after a primary series. In the two clinical studies, plasma was harvested after the second immunization in a primary series. The plasma of volunteers immunized with a J5 vaccine before preparation of an IVIG had only a two- to threefold increase in anti-J5 antibody. Interestingly, Dale and colleagues found that human anti-J5 antisera, obtained from a volunteer at 9 months after immunization, was more active in vitro than sera from the same individual that was harvested earlier in the course of the immunization (18). Thus, the immunization regimen or the time of harvest of anti-J5 plasma after immunization might be an important variable.

The preparation of the vaccine and primary immunization schedule is another variable that must be evaluated more rigorously. Since there is variability in the degree of rough LPS phenotype among single colonies isolated from a culture of LPS, failure to select a sufficiently rough phenotype may result in a less than optimal presentation of a J5 epitope to the immune system. Even when this is taken into account, we found differences in antibody response to the primary immunization following different immunization regimens and with different concentrations of killed bacteria within the same regimen (data not shown). These factors may have contributed to the differences observed between the efficacy in our experimental studies and the recent clinical studies.

Once lots of J5 LPS vaccine are made under GMP conditions, it will be possible to extend these studies to human subjects and to answer these questions directly.

ACKNOWLEDGMENT

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